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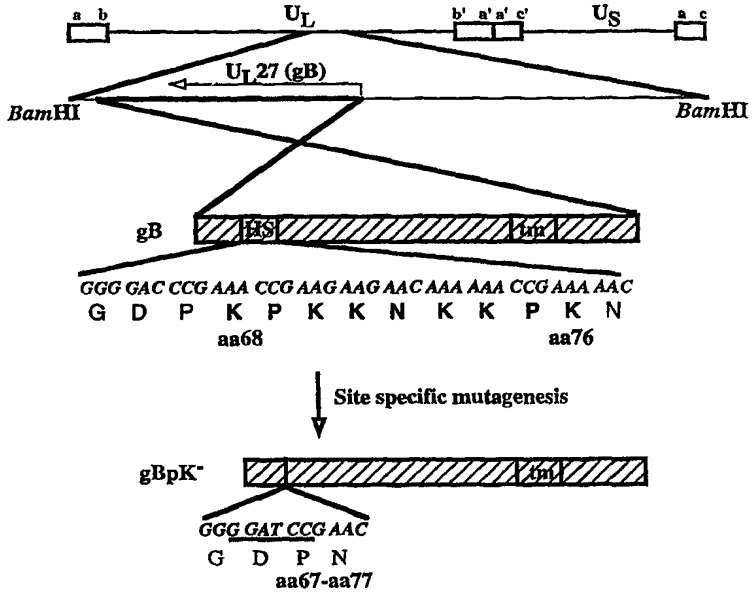

(57) Abstract

The present invention relates to targeting HSV vectors, chiefly by modifying viral proteins. Thus, the present invention provides an HSV having an envelope which includes a non–native ligand. For targeting the HSV vector to a specific cell type, the non–native ligand is typically a cell–surface ligand. Additionally, for altering the native broad targeting of HSV vectors, the targeted HSV can lack a native cell–surface ligand.

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TARGETED HSV VECTORS

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This invention was made with Government support under Grant Number 5R01 CA66141-07 awarded by the National Cancer Institute of the National Institutes of Health. The Government can have certain rights in this invention.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to targeted HSV vectors.

BACKGROUND OF THE INVENTION

Gene transfer technology has wide-ranging utility in a number of applications relating to biological research and the treatment of disease. Central to this technology is a vector for introducing expression cassettes into desired target cells such that genes can be expressed in the target cells.

A particularly attractive vector system employs recombinant herpes simplex virus (HSV) vectors. The HSV viral genome is well characterized as is its life cycle, and the functions of more than 80 native coding polynucleotides are largely defined. One salient feature of HSV vectors is that they can be induced to enter a latent stage by inactivating immediate early genes required for replication (DeLuca et al., *J. Virol.*, *56*, 558 (1985)). During this latent phase, the virus persists in an episomal form for the life of its host (Mellerick and Fraser, *Virology*, *158*, 265-75, (1987); Rock and Fraser, *J. Virol.*, *55*, 849-52, (1985)), neither interfering with cellular function nor inducing autoimmune response (Ramakrishnan et al., *J. Virol.*, 68, 1864-70 (1994); Fruh et al., *Nature*, *375*, 415 (1995)). Furthermore, because the HSV genome is so well characterized, it is readily manipulated for use as a gene transfer vector, a feature enhanced by the fact that HSV genes are generally contiguous linear sequences. Furthermore, as roughly half of its genes are dispensable for growth, the possibility exists of deleting large segments of the HSV genome to accommodate transgenic material (Roizman & Sears, *supra*; Glorioso et al., in *Viral Vectors*, Academic Press, New York (Kaplitt & Loewy, eds.) 1-23 (1995)). Theoretically, up to 30 kb of the HSV genome can thus be replaced with exogenous material without requiring complementary host cells for propagation of the virus.

Primary HSV infection begins by introducing the virus into a host cell, a process involving two distinct stages: attachment of the virus to the cell surface and fusion of the viral envelope with the cell membrane. The virus can also infect cells

by moving transcellularly, (e.g., at the sites of gap junctions), a process referred to as lateral spread. Once the virus has entered a cell, it is transported to the nucleus, whereupon the viral DNA is released and transcribed in three distinct phases of lytic infection.

The attachment and fusion steps of HSV infection are mediated primarily by components of the viral envelope, a membranous structure containing at least 10 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gL, and gM) and four non-glycosilated integral membrane proteins (U_L20, U_L34, U_L45, and $U_L49.5$). Of the glycoproteins, gB, gD, gH, and gL are essential for wild type herpes viruses to infect their host cells (Cai et al., *J. Virol., 62*, 2596-2604 (1988); Desai et al., *J. Gen. Virol., 69* 1147-56 (1988); Hutchinson et al., *J. Virol., 66*, 2240-50 (1992); Ligas et al., *J. Virol., 62*, 1486-94 (1988)), while the remainder are dispensable for viral attachment or internalization (Spear, *Sem. Virol., 4*, 167-80 (1993)); Spear, p. 201-32, *in* "Viral Fusion Mechanisms" (J. Bentz, ed.) (1993 CRC Press, Boca Raton); Steven et al., *Herpesvirus Capsid Assembly and Envelopment*, *in* "Structural Biology of Viruses" (1997, Oxford Univ. Press, New York)). The process of lateral spread to neighboring cells also involves the envelope proteins; however different proteins appear to be essential for each process. Thus, for example, while gE, gG, gI, and gM are not essential for primary infection at the cell surface, removal of any of these greatly inhibits lateral spread (Dingwell et al., *J. Virol., 68*, 834-35 (1994); Dingwell et al., *J. Virol., 69* (1995); Sears et al., *Proc. Nat. Acad. Sci. USA, 88*, 5087-91 (1991)).

The HSV gB glycoprotein is present in the viral envelope as a functional homodimer (Claesson-Welsh et al., *J. Virol., 60*, 803-06 (1986); Highlander et al., *J. Virol., 65*, 4275-83 (1991); Laquerre et al., *J. Virol., 70*, 1640-50 (1996)). The gB protein functions as a ligand for cell-surface glycosaminocglycans (GAGs), notably heparin sulfate (HS) and dermatin sulfate (Herold et al., *J. Gen. Virol., 75*, 1211-22 (1994); Li et al., *J. Virol., 69*, 4758-68 (1995)). In addition to cellular GAG binding, however, gB is required for fusion of the viral envelope with the target cell (Cai et al., ***supra***).

The gC glycoprotein is present in the viral envelope as a monomer. The gC protein functions as a ligand for cell-surface GAGs, notably HS (e.g., Dolter et al., *J. Virol., 66*, 4864-73 (1992); Herold et al., *supra*;), and is considered the primary mediator of viral attachment (Herold et al., *J. Virol., 65*, 1090-98 (1991)). The domain responsible for binding the HS domain has been localized to two separate regions within antigenic site II of the protein (Trybala et al., *J. Gen. Virol., 75*, 743-52 (1994)). Despite its role in cell binding, gC is not required for fusion of the viral

envelope with the cell and subsequent internalization (see, e.g., Herold (1994), *supra*).

The gD glycoprotein is present as a monomer in the HSV envelope. The gD protein functions in viral attachment subsequent to gB/gC mediated binding to cell-surface GAGs by binding a cell-surface protein (HVEM) reported to be a member of the TNFα/NGF receptor family (Montgomery et al., *Cell, 87*, 427-36 (1996)). The native HVEM ligand of gD is localized to the external domain of the protein within residues 1-275 (Whitbeck et al., *J. Virol., 71*, (1997)). Evidence that viruses lacking gD can bind cells but are defective for subsequent penetration, or that the presence of antisera recognizing gD can neutralize attached virus (Fuller et al., *J. Virol., 55*, 475-82 (1985)), provides strong support for a role for gD in the internalization stage of HSV infection. However, these studies have not addressed the possibility that tight binding of gD to the cell surface might potentiate internalization via other proteins (e.g., gB) without direct involvement of gD in the internalization process.

Glycoproteins gE and gI form heterodimers in the viral envelope; dimerization is mediated through interactions between the external domains of the proteins (Basu et al., *J. Immunol., 154*, 260-67 (1995); Johnson et al., *J. Virol., 61*, 2208-16 (1987); Whitbeck et al., *J. Virol., 70*, 7878-84 (1996)). In addition to their role in mediating lateral spread, the heterodimers bind the Fc portion of IgG (Basu et al., *J. Immunol., 158*, 209-15 (1997); Dubin et al., *J. Virol., 68*, 2478-85 (1994)).

As mentioned, the primary agents mediating the binding of HSV for its target cells (i.e., the gB and gC glycoproteins) are ligands for cellular GAGs, notably HS. It is, in part, because the virus infects via this almost ubiquitous cell-surface moiety that wild-type HSV can infect almost any cell type. While native HSV targeting is thus extremely broad, in many gene transfer applications, it is highly desirable to target the transgene to a predefined cell type, or to confine gene transfer to a narrow class of cells or tissues. Despite the many advantages of HSV vectors in gene transfer applications iterated above, this inability to target HSV to desired cell types stands as a major impediment to more widespread use of HSV vectors in therapy and research. Therefore, the state of the art presents a need for a targeted HSV vector.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to targeting HSV vectors, chiefly by modifying viral proteins. Thus, the present invention provides an HSV having an envelope which includes a non-native ligand. For targeting the HSV vector to a specific cell type, the non-native ligand is typically a cell-surface ligand. Additionally, for

altering the native broad targeting of HSV vectors, the targeted HSV can lack a native cell-surface ligand.

The present invention is useful in therapy, for example, by providing vectors for delivering therapeutic genes to cells with minimal ectopic infection. Specifically, the present invention permits more efficient production and construction of safer vectors for gene therapy applications. The present invention is also useful as a research tool in the study of HSV attachment and infection of cells. Similarly, the chimeric proteins and other aspects of the invention can be used in receptor-ligand assays and as adhesion proteins *in vitro* or *in vivo*. Additionally, the present invention provides reagents and methods permitting biologists to investigate the cell biology of viral growth and infection. Thus, the vectors of the present invention are highly useful in biological research. Accordingly, for such medical and scientific uses, the present invention provides stocks of targeted viruses and compositions incorporating such viruses or stocks.

These and other advantages of the present invention, as well as additional inventive features, will be apparent from the accompanying drawings and in the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the genetic manipulation of the HSV gB gene to remove its native HS ligand (SEQ ID NO:1 and SEQ ID NO:2) and produce the gBpK$^-$ allele.

Figure 2 is a schematic representation of the genetic manipulation of the HSV genome to remove the gC gene from a virus having the gBpK$^-$ allele to create the KgBpK$^-$gC$^-$ virus.

Figure 3 is a graphical representation of data pertaining to cell surface binding of viruses including KgBpK$^-$ and KgBpK$^-$gC$^-$.

Figure 4 is a graphical representation of data pertaining to percentage of bound virus/input for viruses including KgBpK$^-$ and KgBpK$^-$gC$^-$.

Figure 5 is a graphical representation of data pertaining to plaque forming ability of viruses including KgBpK$^-$ and KgBpK$^-$gC$^-$.

Figure 6 is a schematic representation of the genetic manipulation of the gBpK$^-$ allele to introduce a non-native ligand (α-bungarotoxin (SEQ ID NO:3 and SEQ ID NO:4)) and produce the chimeric gBpK$^-$BTX allele.

Figure 7 is a graphical representation of data comparing the infectivity of wild type virus and viruses having the gBpK$^-$BTX allele as measured by HSV ICP4 production.

Figure 8 is a schematic representation of the genetic manipulation of the gBpK$^-$ allele to introduce gCEPO allele, encoding a non-native ligand (erythropoeitin), into the virus.

Figure 9 is a graphical representation of data pertaining to binding affinity of viruses having the gBpK$^-$BTX allele as measured by collection of viruses from a substrate-conjugated support.

Figure 10 is a graphical representation of data pertaining to binding of viruses including viruses having the gC:EPO allele.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to targeted HSV vectors. As used herein, targeting generally refers to altering the host-specificity of the virus so that it can more readily infect a set of cell types other than its native set of cell types. In this respect, a selectively targeted virus infects the novel set of cells to a greater degree than wild-type virus, and preferably infects its native set of target cells to a lesser degree than wild type virus.

An HSV vector can be targeted by introducing a novel cell-surface ligand into the viral surface (e.g., the HSV envelope). For targeting the vector to a cell type other than that naturally infected (or a group of cell types other than the natural range of host cells), the non-native ligand can bind to a cell surface binding site (e.g., any site present on the surface of a cell with which the ligand can interact to bind the cell and thereby promote cell entry) other than a native HSV binding site. The nonnative ligand can be such that it enables direct or indirect binding of the virus to the desired cell-surface binding site. Direct binding is mediated where the ligand itself recognizes the binding site. Indirect binding is mediated through an intermediary agent, such as a bispecific or multispecific molecule (e.g., an antibody,) recognizing both the non-native ligand on the viral envelope and the cell surface binding site.

A cell surface biding site can be any suitable type of molecule, but typically is a protein (including a modified protein), a carbohydrate, a glycoprotein, a proteoglycan, a lipid, a mucin molecule or mucoprotein, or other similar molecule. Examples of potential cell surface binding sites include, but are not limited to: GAGs; sialic acid moieties found on mucins, glycoproteins and gangliosides; common carbohydrate molecules found in membrane glycoproteins, including mannose, N-acetyl-galactosamine, N-acetyl-glucosamine, fucose, and galactose; glycoproteins such as ICAM-1, VCAM, E-selectin, P-selectin, L-selectin, and integrin molecules; neurotransmitter receptors (e.g., cholinergic receptors, adrenergic receptors, etc.); and tumor-specific antigens present on cancerous cells (e.g., MUC-1 tumor-specific

epitopes). While the substrates for many cell-specific ligands are proteins naturally present on the surface of a given class of cells, other cells can be engineered to express the appropriate substrate for the non-native ligands (e.g., cells engineered to express single chain antibodies (ScAbs), FAB fragments, or other substrates of interest). The substrate can thus be expressed in a narrow class of cell types (e.g., tumor cells, neuronal cell types, muscle cells types, etc.) or expressed within a broader group encompassing several cell types.

The HSV of the present invention can include any suitable non-native ligand (e.g., a peptide specifically binding to a substrate). Examples of suitable ligands and their substrates for use in the invention include, but are not limited to: CR2 receptor binding the amino acid residue attachment sequences, CD4 receptor recognizing the V3 loop of HIV gp120, transferrin receptor and its ligand (transferrin), low density lipoprotein receptor and its ligand, the ICAM-1 receptor on epithelial and endothelial cells in lung and its ligand, erythropoeitin (EPO) and its receptor, the ligand domain from α-bungarotoxin (SEQ ID NO:4) and its substrate (α-bungarotixin), somatostatin (SST) and its single chain receptor (SEQ ID NO:5), linear or cyclic peptide ligands for streptavidin or nitrostreptavidin (Katz, *Biochemistry*, *34*, 15421 (1995)), and asialoglycoproteins that recognize deglycosylated protein ligands. Moreover, additional ligands and their binding sites preferably include (but are not limited to) short (e.g., 6 amino acid or less) linear stretches of amino acids recognized by integrins, as well as polyamino acid sequences such as polylysine, polyarginine, etc. Also, a ligand can comprise a commonly employed peptide tag (e.g., short polypeptide sequences known to be recognized by available antisera) such as sequences from glutathione-S-transferase (GST) from *Shistosoma manosi*, thioredoxin β-galactosidase, or maltose binding protein (MPB) from *E. coli.*, human alkaline phosphatase, the FLAG epitope, hemagluttinin (HA), polyoma virus peptides, the SV40 large T antigen peptide, BPV peptides, the hepatitis C virus core and envelope E2 peptides, the c-myc peptide, adenoviral penton base epitopes (Babco), epitopes present in the E2 envelope of the hepatitis C virus (see, e.g., Chan, *J. Gen. Virol.*, *77*, 2531 (1996)), and other commonly employed tags. A preferred substrate for a tag ligand is an antibody directed against it, a derivative of such an antibody (e.g., a FAB fragment, ScAb, etc.), or other similar substrate molecule.

Once a given ligand is identified, it can be incorporated into any location of the HSV surface capable of interacting with a substrate (e.g., the external domain of an envelope protein, the tegument, the envelope membrane, structural proteins, etc.). Preferably, however, the ligand is attached to an envelope protein, as viral-cell interaction is naturally mediated via such proteins as described herein. Where the

non-native ligand is added to an envelope protein, preferably it is incorporated in such a manner as to be readily exposed to the substrate (e.g., at the external amino- or carboxy-terminus of the protein, attached to a residue facing the substrate, positioned on a peptide linker or spacer to contact the substrate, etc.) in order to maximally present the ligand to the substrate.

The non-native ligand can be attached to the viral envelope by any means. For example, the non-native ligand can be chemically crosslinked to the surface of the virus. Methods of attaching moieties to the surfaces of HSV viruses are known in the art and within the purview of a skilled artisan. Because many ligands are polypeptide species and because, as mentioned, the envelope proteins are preferred sites for attachment of the non-native ligands, such ligands are preferably attached to the virus by engineering variations into HSV envelope proteins such that the ligands form novel domains of the modified (i.e., chimeric) proteins. Thus, the present invention provides an HSV incorporating a chimeric envelope protein comprising a non-native ligand. Of the envelope proteins, the HSV envelope glycoproteins are naturally positioned in the envelope suitably for contact with extraviral surfaces such as cell surfaces. Therefore, chimeric envelope proteins more preferably involve native glycoproteins (i.e., the chimeric proteins are mutant forms of proteins which are glycoproteins in their native state). Because, as mentioned herein, gB, gC, and gD each mediate viral attachment to the cell surface, chimeric proteins of the present invention are most preferably mutant forms of gB, gC, and/or gD.

When engineered into a chimeric protein, the ligand can comprise a portion of the native sequence in part and a portion of the non-native sequence in part. Similarly, the sequences (either native and/or nonnative) that comprise the ligand in the chimeric protein need not necessarily be contiguous in the chain of amino acids that comprise the protein. In other words, the ligand can be generated by the particular conformation of the protein, (e.g., through folding of the protein) in such a way as to bring contiguous and/or noncontiguous sequences into mutual proximity.

The chimeric protein including the ligand can include other non-native elements as well. For example, as mentioned, the ligand can be incorporated into the chimeric protein attached to a linker or spacer polypeptide. Such a spacer permits, for example, the ligand to be added to the protein without appreciably perturbing the overall protein structure. Moreover, a non-native, unique protease site also can be inserted into the polypeptide sequence of the chimeric protein (e.g., as a separate domain of the protein or as a sequence within a spacer or linker polypeptide). Many such protease sites are known in the art; for example, thrombin recognizes and cleaves at a known amino acid sequence (Stenflo et al., *J. Biol. Chem.*, *257*, 12280-90

(1982)). The presence of such a protease recognition sequence permits, for example, the ligand to be severed from the protein, which can be advantageous in some applications.

Depending upon the degree of targeting desired, the HSV of the present invention can include more than one (e.g., two or more) non-native ligands as described herein. For example, an HSV can include several species of chimeric proteins derived from the same native glycoprotein (e.g., one or more types of gB, gC, gD, etc.). Furthermore, the HSV can include several types of chimeric proteins derived from different envelope proteins. Moreover, each species of chimeric protein can have a different ligand, thus targeting the HSV to several cell types. Alternatively, where cell-surface substrates are not prevalent on the surface of target cells, the non-native ligands of the chimeric proteins can recognize different binding sites present on the same cell type, thus boosting infectivity. Furthermore, where the HSV envelope includes more than one type of chimeric protein, the non-native ligands can bind to the same substrate, thus increasing the affinity of the virus for that substrate.

In addition to mediating novel targeting, the HSV of the present invention preferably is further modified to decrease native targeting. Thus, the viral envelope preferably lacks a native cell-surface ligand. As mentioned herein, the native cell-surface ligands (e.g., recognizing HS, HVEM, etc.) are present on envelope proteins. Thus, in one embodiment, the viral envelope lacks one or more of the proteins having a native cell-surface ligand. For example, a substantial reduction in HS affinity is effected by removing gC from the envelope. Moreover, where the viral envelope includes chimeric gC and gB proteins, each having high-affinity ligands recognizing the same substrate, the envelope can lack gD without ablating viral internalization.

Alternatively, to reduce the natural broad HSV targeting, the HSV preferably comprises at least one mutant envelope protein wherein its native cell-surface ligand has been inactivated. For example, while gC is the primary protein mediating HSV binding, gB and gD also have native cell-surface ligands, as mentioned. Thus, the mutant envelope protein preferably is gB, gC, or gD wherein the native cell-surface ligand has been inactivated.

One means of inactivating a native ligand is via inclusion of a non-native polypeptide domain blocking the native cell-binding ligand. The blocking polypeptide is any peptide which can be tightly bound to the native ligand (See, e.g., Hong et al., *EMBO J.*, *16*, 2294-2306 (1997)). In other words, the blocking polypeptide is a substrate to which the native ligand selectively binds. For example, to bind to the ligand of gB or gC, the blocking polypeptide can present heparin-like

sequences able to bind the polycationic ligands of these proteins. Generally, the ligand-blocking substrate interaction occurs at least immediately upon viral production and effectively continues for the life of the protein (or until such blocking polypeptide is otherwise removed or inactivated, e.g., via protease cleavage as described herein). Because the native ligand binds the blocking polypeptide, its ability to bind its native substrate on cell surfaces is attenuated or substantially ablated. The blocking polypeptide can be at any position on the virus to bind the native ligand. The blocking polypeptide also can include a linker or spacer domain to facilitate its interaction with the native ligand; of course, such linkers or spacer domains can include protease recognition sites, as described herein, to facilitate preferential removal of the blocking polypeptide domain, if desired.

Another means of inactivating the native ligand is to remove the ligand sequence from the protein. Thus, a mutant envelope protein can incorporate any suitable modification removing its native ligand domain. For example, the mutant protein also can be a chimeric protein including a non-native ligand, as described herein. In such instances, the non-native ligand can replace the native cell surface ligand domain. Alternatively, native cell-surface ligands can be removed by deleting domains comprising the ligands. For example, as gC is completely dispensable for HSV attachment, its large external domain can be substantially removed.

Another means of inactivating a native ligand is to alter its topology, for example, by introducing one or more amino acid residues (e.g., a spacer sequence) into the native ligand. Similarly, the ligand domain can be inactivated by altering its charge. For example, the ligands of gB and gC are generally polycationic (including several lysine or arginine residues, respectively) and are believed to interact with their substrate largely via ionic interactions (see Trybala et al., *supra*). Thus, one means of inactivating the gB or gC HS ligand domains is by introducing amino acid substitutions (e.g., by site specific mutagenesis) altering their cationic charge.

Where recombinant proteins are included in HSV envelopes, preferably the mutations do not significantly alter essential functions of the proteins other than targeting. For example, because of the essential roles of gB and gD in viral infection, mutations affecting these two proteins preferably do not substantially reduce the ability of these proteins to mediate fusion of the viral envelope and cell membrane. Thus, for example, where the HS-binding residues of gB (i.e., residues 68-76 (SEQ ID NO:2), see Figure 1) is removed and replaced with a non-native amino acid sequence, preferably the size of the non-native sequence does not appreciably alter the overall protein topology. Such mutant gB proteins, while unable to mediate HS binding, are still able to direct viral-cell fusion. The HVEM-binding domain of gD

can be similarly manipulated to attenuate native HSV targeting but preserve the protein's ability to mediate viral-cell fusion. Moreover, as recombinant envelope proteins must correctly associate into the HSV envelope, manipulation of protein structure preferably does not appreciably perturb insertion into the membrane, oligomerization, or other parameter involving interaction of the protein with other envelope constituents.

The HSV of the present invention can comprise any combination of proteins, membrane, and other constituents such that the structural integrity of its envelope is preserved. To reduce native HSV targeting and promote novel targeting, however, the HSV envelope preferably consists essentially of membrane and proteins selected from the group consisting of native HSV envelope proteins, chimeric envelope proteins having non-native ligands (as described herein), and mutant envelope proteins lacking native ligands (as described herein). Of course, such envelopes also can be deficient for one or more native proteins, such as native HSV envelope glycoproteins. Indeed, as described herein, many of these proteins are not essential for viral-cell fusion and can be removed from the viruses of the present invention.

As mentioned, the HSV of the present invention has a non-native ligand, preferably recognizing a substrate on the surface of a cell. While the ligand can bind the substrate with any affinity, the combination of ligand-substrate interactions between the virus and the cell surface permits the HSV to bind the surface of a target cell. Where the HSV also lacks native cell-binding ligands (e.g., HS, HVEM, etc.) as herein described, the affinity of the HSV for its native set of host cells is substantially reduced, and preferably an HSV of the present invention does not selectively bind to all cell types within the set of natural HSV host cells. This concomitant decrease in broad native targeting and introduction of novel targeting effects selective targeting of the HSV of the present invention. However, where the HSV is to serve as a vector to deliver a genome to a target cell, the virus preferably can be internalized into the cell (e.g., through the presence of functional gB and/or gD fusion domains or via inclusion of a novel mediator of cell-viral fusion).

Because the virus can bind to and fuse with a defined set of target cells, it is a suitable vector for gene transfer applications, such as for use in biological research or in therapy. Thus, the viruses of the present invention can have one or more transgene expression cassettes (i.e., a polynucleotide for expression operably linked to a promoter, optionally including polyadenylation sequences or other processing sequences). Such transgenes can encode marker proteins, therapeutically active proteins, proteins mediating cell death, proteins for secretion, proteins altering cellular metabolism or physiology, or gene products exerting their effects at the level

of RNA (e.g., ribozymes, antisense RNA, etc.). The choice of transgene is within the purview of those skilled in the art and will largely depend upon the use to which the targeted HSV vectors are put.

Generally, the HSV of the present invention is most useful when enough of the virus can be delivered to a cell population to ensure that the cells are confronted with a certain number of viruses. Thus, the present invention provides a stock of targeted HSV. Viral stocks vary considerably in titer, depending largely on viral genotype. Furthermore, for delivery into a host (such as an animal), an HSV, or viral stock, of the present invention can be incorporated into a suitable carrier. As such, the present invention provides a composition comprising an HSV of the present invention and a pharmacologically acceptable carrier. Any suitable preparation is within the scope of the invention; the exact formulation, of course, depends on the nature of the desired application (e.g., cell type, mode of administration, etc.), and is within the purview of those of skill in the art.

As described herein, the HSV can include recombinant proteins having exogenous cell-surface ligands and/or lacking native ligands. Such recombinant proteins can be produced by any suitable manipulation. For example, recombinant proteins can be synthesized using standard direct peptide synthesizing techniques (e.g., as summarized in Bodanszky, *Principles of Peptide Synthesis*, (Springer-Verlag, Heidelberg: 1984)), such as via solid-phase synthesis (see, e.g., Merrifield, *J. Am. Chem. Soc.*, *85*, 2149-54 (1963); Barany et al., *Int. J. Peptide Protein Res.*, *30*, 705-739 (1987); and U.S. Patent 5,424,398). Alternatively, a plasmid, oligonucleotide, or other vector encoding the desired mutation can be recombined with the viral genome, or with an expression vector encoding the native protein, to introduce the desired mutation. Oligonucleotide-directed site-specific mutagenesis procedures (e.g., Walder et al., *Gene*, *42*, 133 (1986); Bauer et al., *Gene*, *37*, 73 (1985); Craik, *Biotechniques*, 12-19 (January 1995); and U.S. Patents 4,518,584 and 4,737,462) are also appropriate.

However engineered, the DNA fragment encoding the recombinant protein (e.g., a chimeric protein having a non-native ligand, a mutant protein lacking a native ligand, etc.) can be subcloned into an appropriate vector using well known molecular genetic techniques. The fragment is then transcribed, and the peptide is subsequently translated *in vitro* within a host cell. Any appropriate expression vector (e.g., as described in Pouwels et al., *Cloning Vectors: A Laboratory Manual* (Elsevior, NY: 1985)) and corresponding suitable host can be employed for production of recombinant peptides. Expression hosts include, but are not limited to, bacterial species, mammalian or insect host cell systems including baculovirus systems (e.g.,

as described by Luckow et al., *Bio/Technology*, *6*, 47 (1988)), and established cell lines such 293, COS-7, C127, 3T3, CHO, HeLa, BHK, etc. Of course, the choice of expression host has ramifications for the type of peptide produced, primarily due to post-translational modification. Once produced, the recombinant proteins can be assayed for their ability to bind the native substrates (e.g., HS, HVEM, etc.) and/or the substrate appropriate for the non-native ligand (if present).

A chimeric protein having a non-native ligand or a mutant protein lacking a native cell-surface ligand can be incorporated into an HSV of the present invention by any suitable means. Where an expression cassette encoding the mutant protein (e.g., as described above) is co-introduced into a host cell with a viral genome, the cellular machinery effects the translation of both vectors and the production of HSV having envelopes incorporating the mutant proteins. Preferably, however, to ensure more consistent production of recombinant HSV, the genes encoding the mutant proteins described herein are suitably introduced into the HSV genome in a manner appropriate to effect their expression during lytic infection. Methods of introducing exogenous mutations or expression cassettes into the HSV genome are well-known in the art. A common method of engineering recombinant HSV employs a host cell line to direct homologous recombination between a source HSV and DNA mutating vectors (e.g., plasmid vectors, HSV or other viral vectors, etc.) comprising the desired mutant sequence (e.g., deletion, insertion, or substitution) flanked by sequences homologous to the desired locus within the HSV genome. A single round of homologous recombination within the host cell line can introduce one or several desired mutations into the source HSV, and the resultant viruses can be identified by Southern blotting, assaying for expression of a transgene, or other suitable method. Alternatively, HSV of the present invention can be produced by introducing a novel restriction site into the HSV genome, cutting the genome, and introducing a cassette encoding the mutation at the site of the novel restriction site as described in U.S. Patent Application Serial No. 08/854,601.

The following examples further illustrate the present invention. In particular, they demonstrate modified HSV envelope proteins lacking native cell-surface ligands, chimeric HSV proteins having non-native ligands, and herpes simplex viruses including envelopes containing non-native ligands. The data presented demonstrate that such modifications can target HSV to cells other than the set of cells naturally infected by HSV. Of course, as these examples are included for purely illustrative purposes, they should not be construed to limit the scope of the present invention in any respect.

Several reagents and procedures are common to the examples. Vero and Human Embryonic Lung (HEL) cell lines were obtained from American Type Culture Collection. A Vero cell line (A1), stably transfected with the HSV-1 genes encoding gB and ICP 18.5, was used to propagate a mutant virus (KΔ4BX, Desai, et al., *Virology, 204*, 312-22 (1994)) lacking those genes. A mutant mouse L-cell line (gro2C, Greunheid et al., *J. Virol., 67*, 93-100 (1993)), defective in HS synthesis, was also employed. BOSC23 a cell line stably expressing the gag-pol and env genes of MoMLV cells were employed in some assays. The cell lines were propagated in Dulbecco's modified minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) at 37 ^{0}C. HSV-1 KOS, and a gC$^-$ KOS derivative viruses (gC$^-$39, Hutchinson et al., *J. Virol., 66*, 2240-50 (1992)) were propagated and titered on Vero cells. Mutant HSV-1 viruses were constructed by standard methods for marker transfer using LIPOFECTAMINE™ (Gibco-BRL) for co-transfection. Other reagents and procedures are within the state of the art (see generally Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); Graham et al., Virol., 52, 456-67 (1973)).

EXAMPLE 1

This example demonstrates a modified HSV protein lacking its native ligand.

To create the modified protein, a lysine rich sequence (pK) comprising the putative HS ligand was deleted from the N-terminal region of the gB glycoprotein resulting in a gBpK$^-$ recombinant molecule. The HSV-1 gB coding sequence (U_L27), was excised by enzymatically digesting the BamG fragment of the viral genome with KpnI and SalI endonucleases and sub-cloned in KpnI-XhoI restriction sites of the pTZ18U vector (pTZ18UgB1). As is indicated in Figure 1, site specific mutagenesis was performed to delete the 27 nucleotides (SEQ ID NO:1) encoding the putative HS binding domain of gB (from amino acids 68 to 76 (SEQ ID NO:2)), and a BamHI recognition site was inserted in frame at the site of the mutation. The resulting gBpK$^-$ mutant allele was subsequently inserted into the pKBXX vector (Cai et al., *supra*), to create a plasmid (pgBpK$^-$) having the gBpK$^-$ allele. Both plasmids were further modified by inserting the human cytomegalovirus immediate early promoter (HCMV-IEp) at their 5'-ends to create HCMV-BXX and HCMV-gBpK$^-$.

The ability of the gBpK$^-$ recombinant molecule to be processed and to bind heparin was investigated. To evaluate the post-translational processing of the mutated gB, Vero cells were transfected with expression vectors encoding wild type gB (HCMV-BXX) and gBpK$^-$ (HCMV-gBpK$^-$); mock transfected Vero cells were used as a negative control. Twenty-four hours post-transfection the cells were

analyzed by immunofluorescence for the presence and localization of the gB molecules. Monolayers were incubated with a pool of anti-gB monoclonal antibodies (Marlin et al., *J. Virol., 59*, 142-53 (1986)), rinsed with cold tris buffered saline pH 7.4 (TBS), and incubated for an additional hour with a cys3-conjugated anti-mouse antibody. The monolayers were fixed with 2% paraformaldehyde and processed for photography. Immunofluorescence staining demonstrated that both wild type gB and recombinant gBpK$^-$ were recognized by the monoclonal antibodies, and both proteins were incorporated into the cell surface membrane. No fluorescence was detected from mock transfected control cells.

To investigate the effect of the gBpK$^-$ mutation on HS binding, gB proteins were compared in an HS binding assay. The pKBXX and pgBpK$^-$ expression vectors were used to produce wild type and mutant proteins, respectively, following gB induction after superinfection with KΔ4BX. Twenty four hours post-transfection the cell monolayers were infected at an MOI of 3 with KΔ4BX virus in presence of [^{35}S]methionine/cysteine. Ten hours post-infection, the cell monolayers were scraped, harvested, and resuspended in 200 µl of a 1% TRITON-X100 based lysis buffer. The TRITON soluble extracts were incubated for 2 hours at 4 °C in presence of heparin-acrylic beads. The beads were rinsed 3 times with lysis buffer or lysis buffer supplemented with 10 mg/ml of heparin. The TRITON soluble extracts, as well as lysis buffer washes or lysis buffer supplemented with heparin washes, were immunoprecipitated using a pool of gB antibodies (Marlin et al., *supra*) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Approximately the same quantity of radiolabeled gB or gBpK$^-$ molecules were incubated with the heparin beads. However, the SDS-PAGE analysis demonstrated that the quantity of unbound gBpK$^-$ displayed a lower affinity for heparin-acrylic beads compared to wild type gB glycoprotein; specifically, more than 40% of the gBpK$^-$ molecules were recovered in the unbound fraction compared to less than 3% of the gB wild type molecule. The data further indicate that similar amounts of gB molecules were bound nonspecifically to the heparin beads and could be eluted with lysis buffer. The bound glycoprotein was eluted by washing the beads with lysis buffer supplemented with heparin. The SDS-PAGE results further demonstrated that bound wild type gB was efficiently eluted by heparin washes, while a small amount of gBpK$^-$ was released by heparin wash, likely due to the small quantity of gBpK$^-$ previously bound to the heparin beads.

Together these data demonstrated that the gBpK$^-$ recombinant molecule was incorporated into the cell surface membrane and that a domain responsible for heparin binding had been inactivated by deleting the pK region of gB.

EXAMPLE 2

This example demonstrates a chimeric HSV protein having a non-native ligand for acetylcholine.

The pgBpK$^-$ plasmid described herein was digested with BamH1, and a stretch of 45 nucleotides (SEQ ID NO:3) encoding the 13 amino acid acetylcholine-binding ligand from α-bungarotoxin (SEQ ID NO:4) was inserted at the BamH1 site introduced into pgBpK$^-$ to create the pgBpK$^-$BTX plasmid encoding the gBpK$^-$BTX allele (Figure 6).

The ability of the gBpK$^-$BTX recombinant molecule to be processed and to bind heparin- and acetylcholine-conjugated substrates can be investigated as described in Example 1. Immunofluorescence data will demonstrate that the gBpK$^-$BTX recombinant molecule is processed and inserted into plasma membranes. Incubation with heparin- or acetylcholine-conjugated beads will demonstrate that the gBpK$^-$BTX- recombinant molecule binds with reduced affinity to heparin, similar to gBpK$^-$. However, the data will also demonstrate that gBpK$^-$BTX binds to acetylcholine-conjugated beads with far greater affinity than either gBpK$^-$ or wild-type gB. The data, thus, will demonstrate that gBpK$^-$BTX behaves as HSV gB, except that it is a ligand for acetylcholine instead of HS.

EXAMPLE 3

This example demonstrates a chimeric HSV protein having a non-native ligand for somatostatin.

The pgBpK$^-$ plasmid described herein is digested with BamH1, and a stretch of nucleotides encoding the 14 amino acid somatostatin (SST)-binding ligand (SEQ ID NO:5) is inserted at the BamH1 site (similarly as described in Example 2) introduced into pgBpK$^-$ to create the pgBpK$^-$SST plasmid.

The ability of the gBpK$^-$SST recombinant molecule to be processed and to bind heparin- and SST-conjugated substrates will be investigated as described in Example 1. Immunofluorescence data will demonstrate that the gBpK$^-$SST recombinant molecule is processed and inserted into plasma membranes. Incubation with heparin- or SST-conjugated beads will demonstrate that the gBpK$^-$SST recombinant molecule binds with reduced affinity to heparin, similarly to gBpK$^-$. However, the data will also demonstrate that gBpK$^-$SST binds to SST-conjugated beads with far greater affinity than either gBpK$^-$ or wild-type gB. The data, thus, will demonstrate that gBpK$^-$SST behaves as HSV gB, except that it is a ligand for SST instead of HS.

EXAMPLE 4

This example demonstrates a chimeric HSV protein having a non-native ligand for the erythropoeitin (EPO) receptor.

To create the modified protein, a plasmid containing the U_L44 gene, which encodes gC, was first partially digested with NcoI and then digested with BglII, to remove the HS binding domain. Subsequently, a plasmid containing the EPO gene was amplified by PCR primers creating 5' BglII and 3' NcoI restriction sites. The resulting PCR fragment was ligated into the linearized BglII-Nco gC plasmid to create an expression vector (pgCEPO) encoding the gC:EPO allele (Figure 8).

The pgCEPO vector was transfected into BOSC23 cells to produce the chimeric gC:EPO protein in the presence of [^{35}S]met/cys. Supernatants from the transfection were solubilized and immunoprecipitated with anti-gC or anti-EPO antibodies, and the immune precipitates were separated by SDS-PAGE. The data demonstrated the presence of the gC:EPO fusion molecules within the envelope of the pseudotype particles.

Similar experiments were performed where BOSC 23 cells were co-transfected with a plasmid containing the HCMV IEp-lacZ cassette flanked by the LTR sequences in order to encapsulate a transducing reporter gene. The supernatants of the co-transfected cells were used to infect NIH-3T3 and NIH-3T3 cells bearing the EPO receptor (NIH-3T3EPO^r). Infection of NIH-3T3 and NIH-3T3EPO^r cells was accomplished through the binding/fusion properties of gp70 (env product), while the gC:EPO fusion products were tested for binding to the EPO^r. Binding of gC:EPO fusion proteins to the EPO^r was quantitated by comparing the number of transduced NIH-3T3EPO^r to NIH-3T3 cells. The results indicated that particles expressing the gC:EPO fusion protein transduced at least twice as many NIH-3T3EPO^r cells as NIH-3T3 cells.

The presence of the gC:EPO fusion molecules within the envelope of the pseudotype particles demonstrated that the chimeric proteins were processed and transported to the cell surface for further incorporation within the budding virus envelope. Moreover, that particles having the gC:EPO fusion protein preferentially transduced cells expressing the EPO^r demonstrates that the chimeric protein functions as a ligand for the EPO^r.

EXAMPLE 5

This example demonstrates a chimeric HSV protein having a non-native ligand for the acetylcholine receptor.

Similar to the protocol described in Example 4, a stretch of 45 nucleotides (SEQ ID NO:3) encoding the 13 amino acid acetylcholine-binding ligand (SEQ ID NO:4) from α-bungarotoxin is inserted into the gC gene from which the HS ligand has been excised to create the pgCBTX plasmid encoding the chimeric gCBTX allele.

The ability of the chimeric gCBTX protein to be processed and to bind heparin- and acetylcholine-conjugated substrates is investigated as described in Example 1. Immunofluorescence data, using antisera recognizing gC, will demonstrate that the gCBTX recombinant molecule is processed and inserted into plasma membranes. Incubation with heparin- or acetylcholine-conjugated beads will demonstrate that the gCBTX recombinant molecule binds with reduced affinity to heparin. However, the data will also demonstrate that gCBTX binds to acetylcholine-conjugated beads with far greater affinity than wild-type gC.

Similar to the protocol outlined in Example 4, BOSC 23 cells are co-transfected with a plasmid containing the HCMV IEp-lacZ cassette flanked by the LTR sequences in order to encapsulate a transducing reporter gene. The pseudotype particles are used to infect cells expressing the acetylcholine receptor and control cells not expressing the receptor. The results will demonstrate that particles expressing the chimeric pgCBTX protein preferentially transduces cells expressing the acetylcholine receptor.

The data, thus, will demonstrate that gCBTX behaves as HSV gC, except that it is a ligand for acetylcholine instead of HS. Moreover, that particles expressing the pgCBTX fusion protein preferentially transduce cells expressing the acetylcholine receptor will demonstrate that the chimeric protein functions as a ligand for the acetylcholine receptor.

EXAMPLE 6

This example demonstrates a chimeric HSV protein having a non-native ligand for SST.

Similar to the protocol described in Example 4, a polynucleotide encoding the 13 amino acid acetylcholine-binding ligand (SEQ ID NO:5) from SST is inserted into the gC gene from which the HS ligand has been excised to create the pgCSST plasmid encoding the chimeric gCSST protein.

The ability of the chimeric gCSST protein to be processed and to bind heparin- and SST-conjugated substrates is investigated as described in Example 1. Immunofluorescence data, using antisera recognizing gC, will demonstrate that the gCSST recombinant molecule is processed and inserted into plasma membranes. Incubation with heparin- or SST-conjugated beads will demonstrate that the pgCSST

recombinant molecule binds with reduced affinity to heparin. However, the data will also demonstrate that gCSST binds to SST-conjugated beads with far greater affinity than wild-type gC.

Similar to the protocol outlined in Example 4, BOSC 23 cells are co-transfected with a plasmid containing the HCMV IEp-lacZ cassette flanked by the LTR sequences in order to encapsulate a transducing reporter gene. The resulting pseudotype particles are used to infect cells expressing the acetylcholine receptor and control cells not expressing the receptor. The results will demonstrate that particles expressing the chimeric gCSST protein preferentially transduces cells expressing SST.

The data, thus, will demonstrate that gCSST behaves as HSV gC, except that it is a ligand for SST instead of HS. Moreover, that particles expressing the gCSST fusion protein preferentially transduce cells expressing the SST demonstrate that the chimeric protein functions as a ligand for SST.

EXAMPLE 7

This example demonstrates an HSV having a viral envelope incorporating a mutant HSV protein lacking its native cell-surface ligand. Specifically, the HSV (KgBpK$^-$) incorporates a mutant gB protein lacking its native HS binding domain.

To construct a KgBpK$^-$ recombinant virus, the plasmid DNA pgBpK$^-$ and the viral DNA KΔ4BX were co-transfected on the complementing A1 cell line. The KgBpK$^-$ recombinant virus was selected for growth on Vero cells and screened by Southern blot hybridization using a gB-specific probe.

The probe hybridized to the *Bam*G fragment (7774 bp) of HSV-1 viral DNA containing the wild type gB coding sequence encoded by KOS and the KOS mutant gC$^-$39. This same probe hybridized to a 3009 fragment in the recombinant KgBpK$^-$ viruses. This confirmed the presence of the recombinant gB gene because (as discussed in Example 1) a *Bam*HI recognition sequence was introduced at the site of the pK mutation, resulting in the production of two subfragments (3009 and 4738 bp) after digestion.

EXAMPLE 8

This example demonstrates an HSV having a viral envelope lacking a protein having a native cell-surface ligand and further incorporating a mutant HSV protein lacking its native cell-surface ligand and. Specifically, the HSV (KgBpK$^-$gC$^-$) lacks the gC glycoprotein and incorporates a mutant gB protein lacking its native HS binding domain.

The gC gene was deleted from the backbone of the KgBpK$^-$ mutant virus and replaced with the *lacZ* gene under the control of HCMV IEp (see Figure 2). Following gC$^-$ recombinant virus selection by virus neutralization assay using a pool of gC$^-$specific monoclonal antibodies, the double recombinant KgBpK$^-$gC$^-$ mutant was detected by X-gal staining. The recombinant viral DNAs were digested with *Bam*HI, and analyzed by Southern blot using an *Xho*I-*Eco*RV gC probe.

The probe hybridized to a 6640 bp fragment in KOS and KgBpK$^-$ DNAs containing the wild type gC coding sequence and with a 5040 bp fragment in the gC negative gC$^-$39 viral DNA (Holland et al., *J. Virol., 52*, 566-74 (1984)). The same probe, however, failed to hybridize with KgBpK$^-$gC$^-$ viral DNA, demonstrating that the gC coding sequence was deleted. Moreover, the viral KgBpK$^-$gC$^-$ DNA was also shown to lack the pK sequence of gB since a [^{32}P]labeled gB probe hybridized with a 3009 bp fragment in the recombinant viruses as discussed in Example 7. These data confirmed the isolation of a double recombinant virus deleted for the two HS binding elements (i.e., the pK domain of gB and the entire gC protein).

EXAMPLE 9

This example demonstrates that the gBpK$^-$ allele does not affect the level of incorporation of mutant gB molecules into virion envelopes.

The level of incorporation of the glycoproteins into the recombinant virions was determined by specific immunoprecipitation of radiolabeled solubilized envelopes from wild type (KOS) and the gC$^-$39, KgBpK$^-$, and KgBpK$^-$gC$^-$ mutant HSV strains using pools of monoclonal antibodies specific for gB, gC and gD. The immunoprecipitated complexes were then analyzed by SDS-PAGE.

Wild type gB was immunoprecipitated from the KOS and gC$^-$39 viruses, and gBpK$^-$ was immunoprecipitated from the KgBpK$^-$ and KgBpK$^-$gC$^-$ viruses, demonstrating that the mutant gBpK$^-$ molecule was incorporated into the envelopes of all viruses under study. Wild type gC was present only in KOS and KgBpK$^-$, but it was absent in gC$^-$39 and KgBpK$^-$gC$^-$ virus envelopes. Glycoprotein D was detected in all virion envelope preparations. Moreover, the ratio of the quantity (analyzed by densitometry) of gB or gBpK$^-$ to gD demonstrated that the pK$^-$ mutation in gB did not affect the level of incorporation of the mutant gBpK$^-$ molecules into the virion envelopes.

EXAMPLE 10

This example demonstrates that the pK$^-$ mutation in gB does not affect the ability of mutant gB molecules to mediate viral fusion with the target cell. The

example further demonstrates that the capacity of HSV lacking the HS-binding ligands of both gB and gC to infect its native host cell is severely reduced.

The cell-surface affinity of the KgBpK$^-$ and KgBpK$^-$gC$^-$ viruses was compared to that of KOS and gC$^-$39. The attachment efficiency of the four viruses was assessed on Vero cells, and compared to the binding capacity of KOS on gro2C cells. Data produced from these experiments are indicated in Figure 3. The binding capacity of the single mutant KgBpK$^-$ virus was reduced by approximately 35% compared to the binding capacity of the wild type KOS virus. However, this binding capacity of KgBpK$^-$ was higher than that of a gC$^-$negative virus (gC$^-$39) which displayed a 60% reduction in binding compared to wild type KOS virus. This result demonstrated that the pK region of gB contributes to a lesser extent to virus binding to HS than gC. Analysis of the binding capacity of the double mutant KgBpK$^-$gC$^-$ virus demonstrated that the ability of this mutant to attach to cells was reduced by 80%.

Viruses bound to the cell surface were washed with heparin and the quantity of released virus particles taken as a measure of the bound virus' specifically to HS. At each time point, the average number of plaques produced on heparin-washed monolayers was expressed as a percentage of the average number of plaques produced on complete media-washed monolayers. Data from these experiments are presented in Figure 4. Binding of the KgBpK$^-$ virus was less sensitive (12%) to heparin washes than that of KOS, confirming that an HS binding region of gB had been deleted. Moreover, the binding of the double mutant KgBpK$^-$gC$^-$ virus was highly resistant to heparin washes but only to an extent slightly higher (2.4%) than the resistance demonstrated by gC$^-$39, again showing that the great majority of HS binding activity was attributable to gC. These results confirm that the impaired binding of KgBpK$^-$gC$^-$ was due to the deletion of the native HSV HS ligands. The residual bound virus resistant to heparin washes depended on binding to a non-HS receptor, consistent with the known role of gD in mediating viral binding via the HVEM receptor.

A viral penetration assay was performed to determine the effect of deleting one or both native HSV HS ligands. The rate of virus entry into host cells was determined as the rate at which a virus bound to the cell surface becomes resistant to acid treatment as a consequence of penetration of the virus into the host cell, compared to untreated virus controls. The results of these assays are presented in Figure 5. The data demonstrate that the single mutant KgBpK$^-$ virus displayed normal kinetics of penetration (i.e., similar to wild type virus and slightly higher than the rate of penetration of gC$^-$39). However, penetration of the double mutant KgBpK$^-$ gC$^-$ virus was delayed and reduced by more than 70% after 90 min compared to the

rate of penetration of wild-type KOS virus. These data demonstrate that deletion of both HS domains can significantly reduce the ability of HSV to infect its native host cells.

EXAMPLE 11

This example demonstrates an HSV having a viral envelope incorporating a chimeric HSV protein having a non-native ligand and a recombinant HSV envelope protein lacking a native cell surface receptor ligand.

The pgCEPO expression vector from Example 4 was used for homologous recombination with the KgBpK$^-$gC$^-$ virus to produce the KgBpK$^-$gCEPO mutant HSV having both gBpK$^-$ and gCEPO alleles. Immunoprecipitation of sucrose gradient purified [35S]met/cys labeled virions demonstrated that gCEPO was incorporated into the mature virion envelope. In addition, antibody to EPO was able to neutralize the KgBpK$^-$gCEPO recombinant, further supporting the incorporation of the gC:EPO fusion molecule into HSV-1 virions.

The presence of the gC:EPO fusion proteins in cells infected with KgBpK$^-$gCEPO was detected by immunofluorescence with a monoclonal anti-gC antibody and a polyclonal anti-EPO antibody. Anti-mouse FITC conjugated and anti-rabbit Cy3 conjugated antibodies were used for co-localization of gC and EPO epitopes. The EPO epitope was detected in the recombinant viruses, while the gC epitope was detected in cells infected with both wild-type KOS and KgBpK$^-$gCEPO-infected cells.

A fusion protein encoding GST and the soluble EPO receptor (EREX, provided by Dr. John C. Winkelmann, University of Minnesota) was purified from bacteria after IPTG induction. Glutathione-agarose beads were preabsorbed with EREX, the slurry washed and incubated in presence of KOS, KgBpK$^-$gC$^-$ and the KgBpK$^-$gCEPO viruses to evaluate their affinity for the soluble receptor. Results presented in Figure 9 demonstrate that KgBpK$^-$gCEPO was retained in the slurry preadsorbed with EREX, confirming the binding of the recombinant KgBpK$^-$gCEPO virus to the soluble EPO^r.

EXAMPLE 12

This example demonstrates an HSV having a viral envelope incorporating a chimeric HSV protein having a non-native ligand and lacking a native HSV envelope protein having a cell surface receptor ligand.

The pgBpK$^-$BTX plasmid from Example 2 was recombined into the genome of the kΔ4BX virus to introduce the chimeric gB protein into the viral genome.

Marker rescue of the U_L27 and U_L28 genes resulted in the isolation of the mutant virus KpgBpK$^-$BTX.

EXAMPLE 13

This example demonstrates an HSV having a viral envelope incorporating more than one chimeric HSV proteins, the non-native ligands of each recognizing the same substrate.

The pgBpK$^-$BTX plasmid described in Example 2 and the pgCBTX plasmid described in Example 6 are recombined into the genome of the KgBpK$^-$gC$^-$ virus to produce a virus (KpgBpK$^-$BTXgCBTX) encoding both chimeric envelope proteins. Marker rescue of the U_L27 and U_L28 genes, as well as Southern hybridization to probes recognizing gC, will confirm the presence of the alleles.

EXAMPLE 14

This example demonstrates a method of targeting an HSV vector to a predefined cell other than a native HSV target cell by employing an HSV having a viral envelope incorporating a chimeric HSV protein having a non-native ligand.

QT6 cells and QF18 (which are QT6 cells expressing the acetylcholine receptor) were infected with 1000 pfu of the KgBpK$^-$ and KgBpK$^-$BTX viruses. After 24 hours post infection, the monolayers were fixed with methanol and exposed to a primary antibody recognizing the HSV ICP4 protein and a secondary antibody conjugated to cys-3. The labeled cells were analyzed by fluorescence microscopy and the number of labeled cells quantified to determine the number of infected cells (Figure 7).

Roughly three times more QT6 cells were infected with KgBpK$^-$BTX than with KgBpK$^-$. More than eight times as many QF18 cells were infected with KgBpK$^-$BTX than with KgBpK$^-$. These results indicate that the presence of the BTX ligand conferred a non-specific improvement in binding to cell surfaces over the gBpK$^-$ protein but that the chimeric protein was able to direct selective HSV targeting to cells having the substrate for the non-native ligand.

EXAMPLE 15

This example demonstrates a method of selectively targeting an HSV vector to a predefined cell other than a native HSV target cell by employing an HSV having a viral envelope deficient for a native HSV envelope protein and incorporating a chimeric HSV protein having a non-native ligand.

[35S]met/cys labeled viruses were incubated at 37 oC in presence of K562 cells bearing approximately 800 copies of the EPO^r/cell (Kasahara et al., *Science, 266*, 1373-76 (1994)). At selected time intervals the cells were centrifuged, washed 3 times with complete media and counted for virus associated radioactivity. The percentage of the bound and penetrated virus was determined as the radioactive counts representing the bound and penetrated fraction divided by the input.

These results (Figure 10) demonstrate that the binding/penetration of KgBpK$^-$gCEPO on K562 was improved compared to that of KgBpK$^-$gC$^-$ or KOS. The observation that KgBpK$^-$gC$^-$ bound to NIH3T3 cells nearly as well as KOS reflects the low amount of HS GAGs on this cell line.

These results demonstrate that KgBpK$^-$gCEPO virus selectively binds and infects cells expressing the EPO receptor. Moreover, the binding and infectivity of KgBpK$^-$gCEPO virus is superior to the natural binding and infectivity of wild type KOS virus for this cell line. These results demonstrate that engineering chimeric proteins lacking native cell-surface ligands and having non-native cell-surface ligands can effectively target HSV to a predetermined cell type.

WHAT IS CLAIMED IS:

1. An HSV comprising an envelope, said envelope including a non-native ligand.

2. The HSV of claim 1, wherein said non-native ligand is a cell-surface ligand.

3. The HSV of claim 1 or 2, wherein said non-native ligand is attached to an envelope protein.

4. The HSV of any of claims 1-3, comprising a chimeric envelope protein having said non-native ligand.

5. The HSV of claim 4, wherein said envelope protein is a native glycoprotein.

6. The HSV of claim 4 or 5, wherein said envelope protein is gB, gC, or gD.

7. The HSV of claim 1, wherein said non-native ligand is chemically crosslinked to said envelope.

8. The HSV of any of claims 1-7, comprising two or more of said non-native ligands.

9. The HSV of claim 8, wherein said non-native ligands recognize binding sites present on the same cell type.

10. The HSV of claim 8 or 9, wherein said non-native ligands bind to the same substrate.

11. The HSV of any of claims 1-10, which lacks a native cell-surface ligand.

12. The HSV of claim 11, further comprising a mutant envelope protein which lacks its native cell-surface ligand.

13. The HSV of claim 12, wherein said envelope protein is gB, gC, or gD.

14. The HSV of claim 12 or 13, wherein said non-native ligand replaces said native cell surface ligand.

15. The HSV of any of claims 1-14, which consists essentially of envelope membrane and proteins selected from the group consisting of native HSV envelope proteins, said chimeric envelope proteins, and said mutant envelope proteins.

16. The HSV of any of claims 1-15, which is deficient for a native HSV envelope protein or glycoprotein.

17. The HSV of any of claims 1-16, which binds to the surface of a cell.

18. The HSV of claim 17, which can be internalized into a cell.

19. The HSV of any of claims 1-18, further comprising a transgene.

20. A stock comprising the HSV of any of claims 1-19.

21. A composition comprising the HSV of any of claims 1-19 and a pharmacologically acceptable carrier.

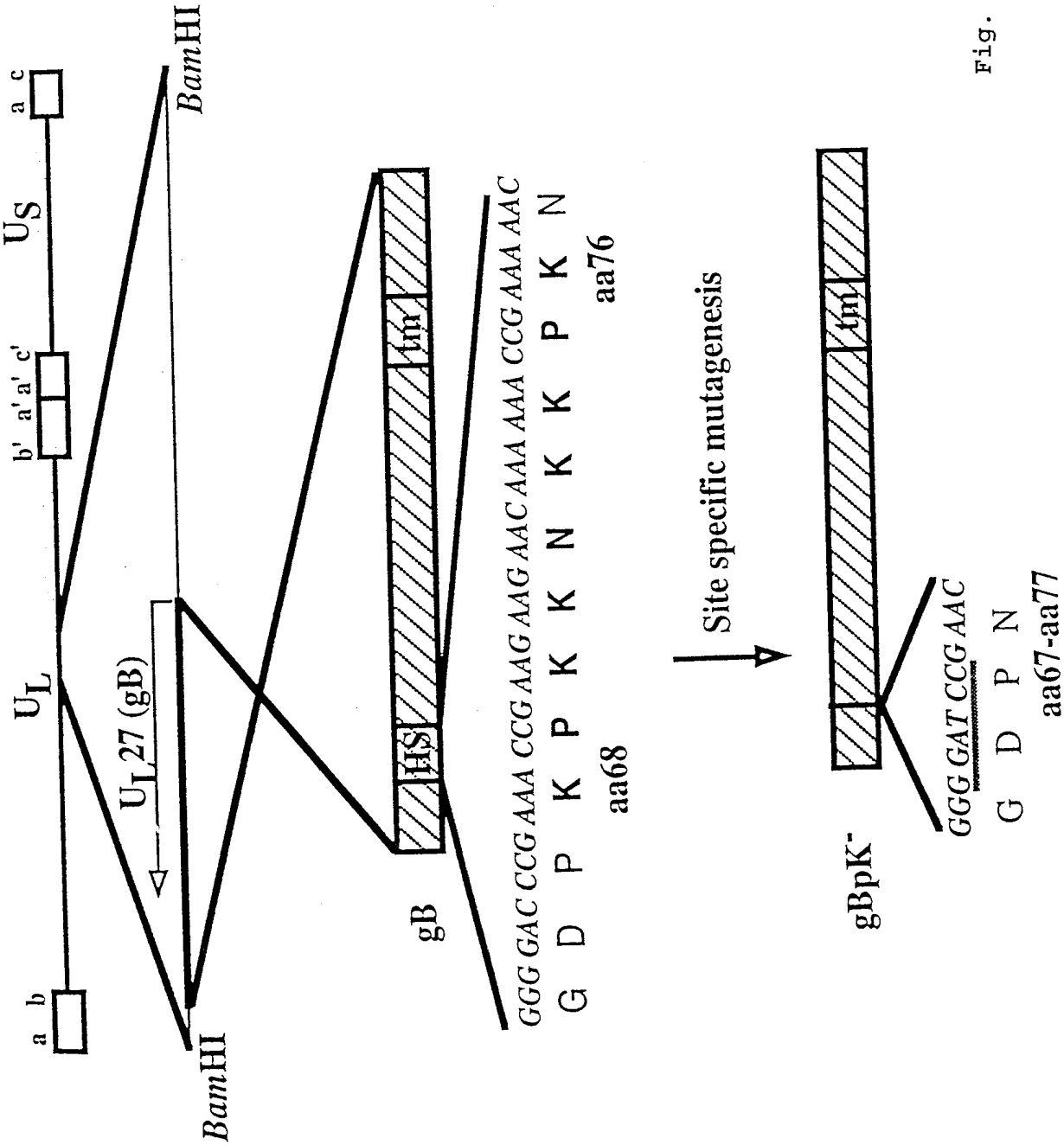
a b
b' a' a' c'
a c
U_L
U_S
U_L27 (gB)
BamHI
BamHI
gB
HS
tm
GGG GAC CCG AAA CCG AAG AAG AAC AAA AAA CCG AAA AAC
G D P K P K K N K K P K N
aa68
aa76
Site specific mutagenesis
gBpK-
tm
GGG GAT CCG AAC
G D P N
aa67-aa77

Fig. 1

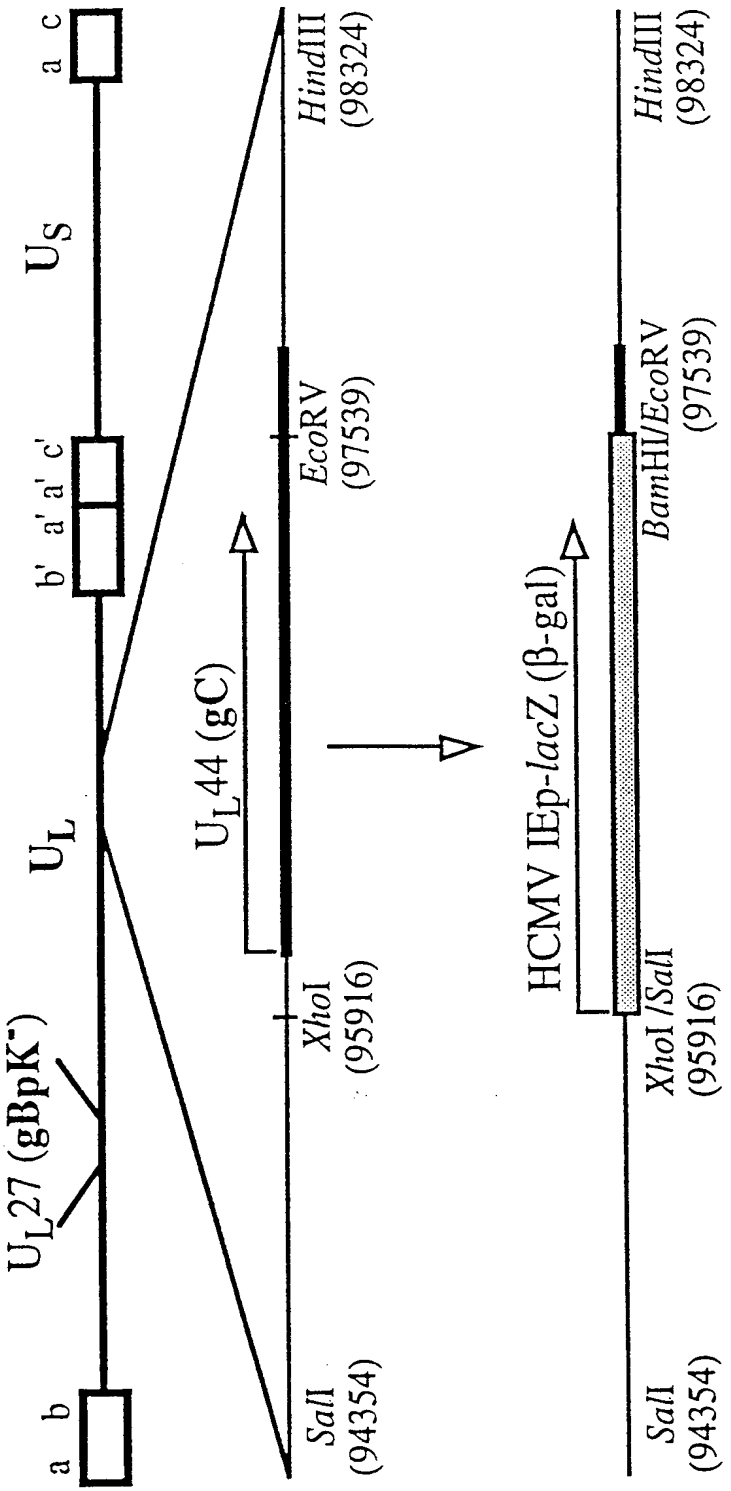
a b
U_L27 (gBpK⁻)
U_L
U_S
a c
b' a' a' c'
U_L44 (gC)
SalI (94354)
XhoI (95916)
EcoRV (97539)
HindIII (98324)
HCMV IEp-lacZ (β-gal)
SalI (94354)
XhoI /SalI (95916)
BamHI/EcoRV (97539)
HindIII (98324)

Fig. 2

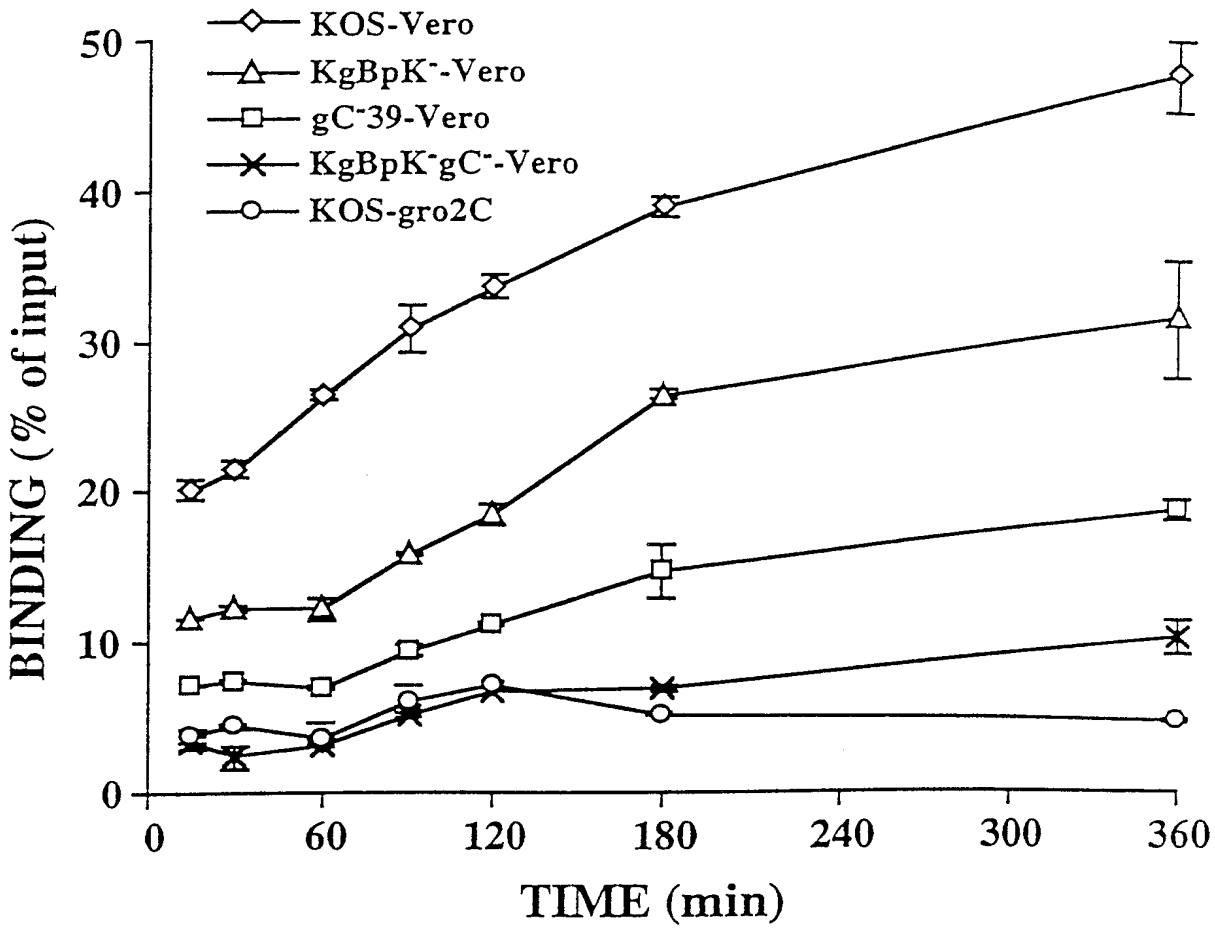
KOS-Vero
KgBpK⁻-Vero
gC⁻39-Vero
KgBpK⁻gC⁻-Vero
KOS-gro2C
BINDING (% of input)
0
10
20
30
40
50
0
60
120
180
240
300
360
TIME (min)

Fig. 3

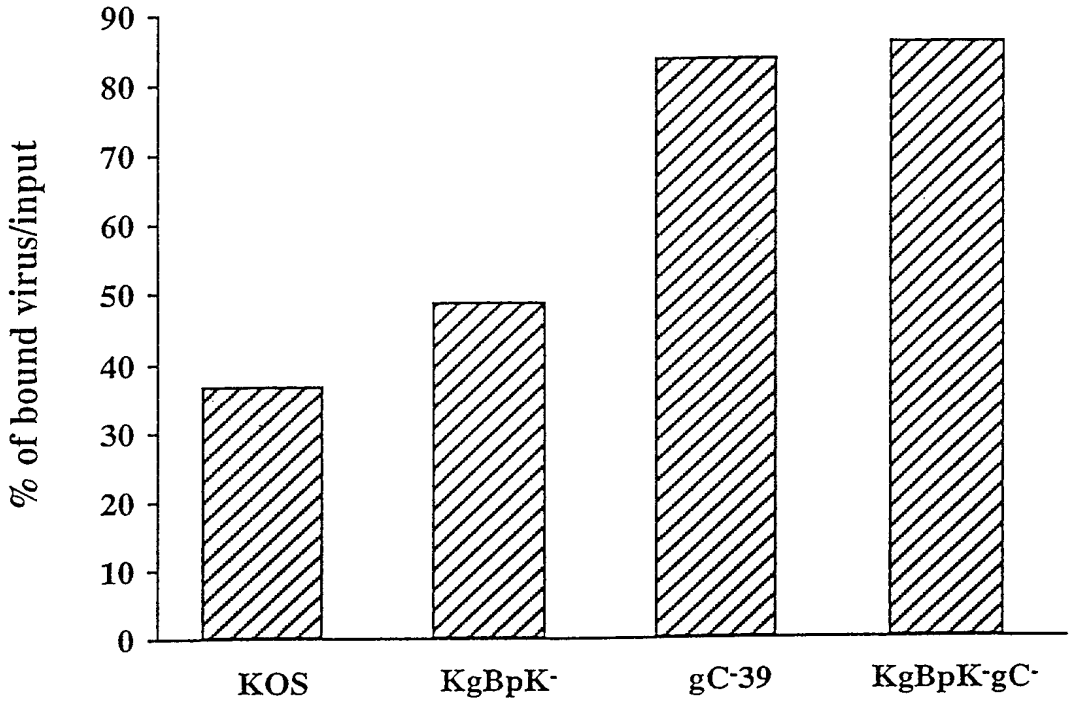
% of bound virus/input
90
80
70
60
50
40
30
20
10
0
KOS
KgBpK-
gC-39
KgBpK-gC-

Fig. 4

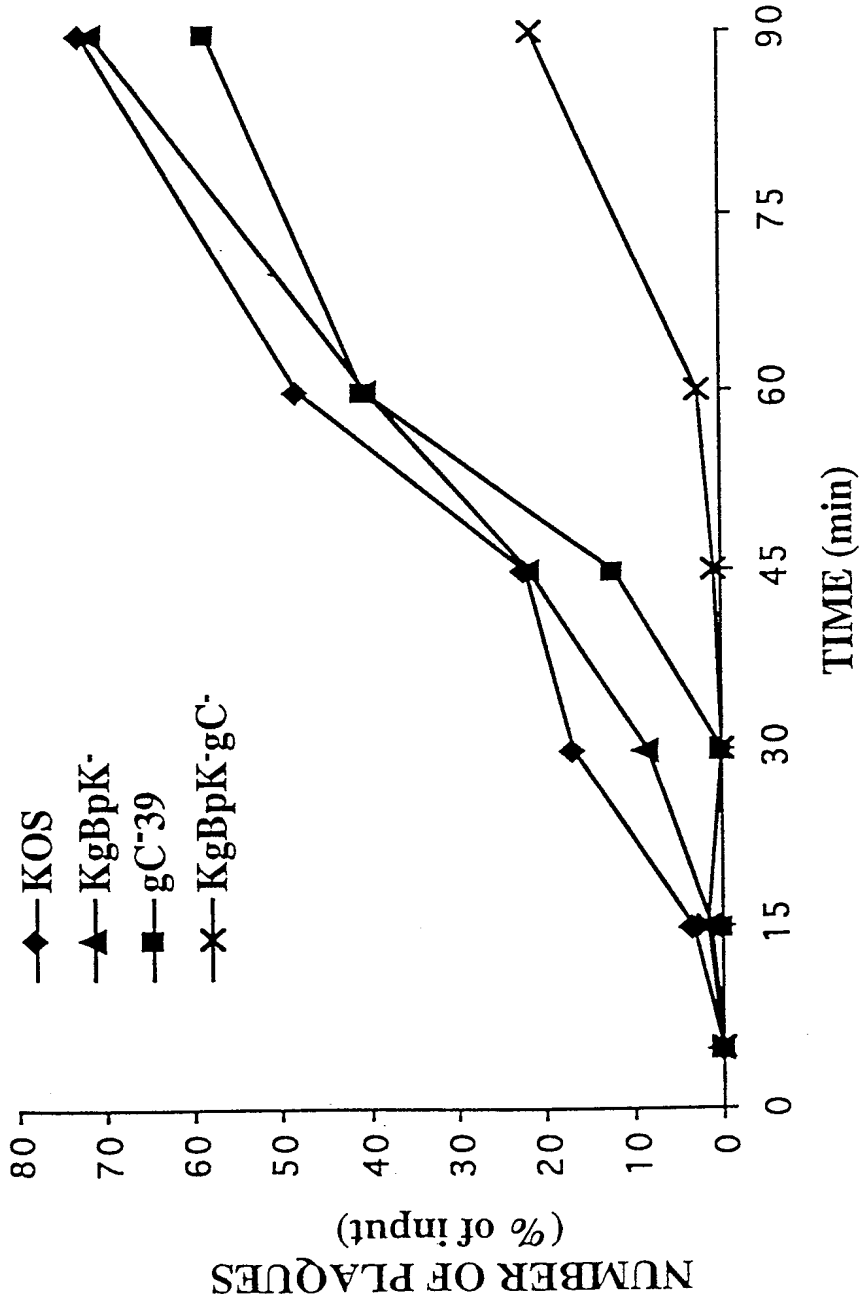
NUMBER OF PLAQUES
(% of input)
80
70
60
50
40
30
20
10
0
0
15
30
45
60
75
90
TIME (min)
KOS
KgBpK-
gC-39
KgBpK-gC-

Fig. 5

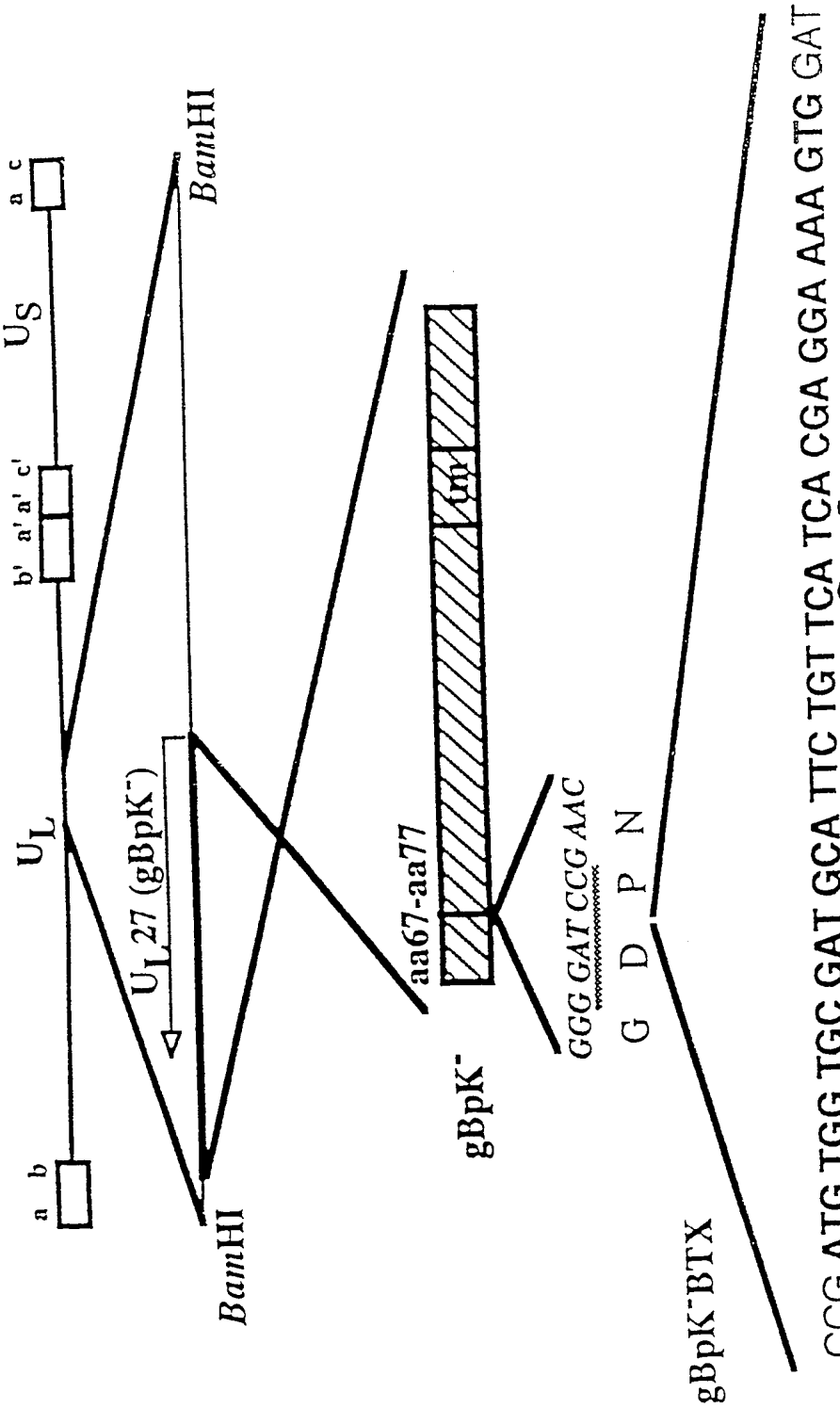
a b
a c
U_L
U_S
b' a' a' c'
U_L27 (gBpK⁻)
BamHI
BamHI
aa67-aa77
gBpK⁻
GGG GAT CCG AAC
G D P N
gBpK⁻BTX
CCG ATG TGG TGC GAT GCA TTC TGT TCA TCA CGA GGA AAA GTG GAT

Fig. 6

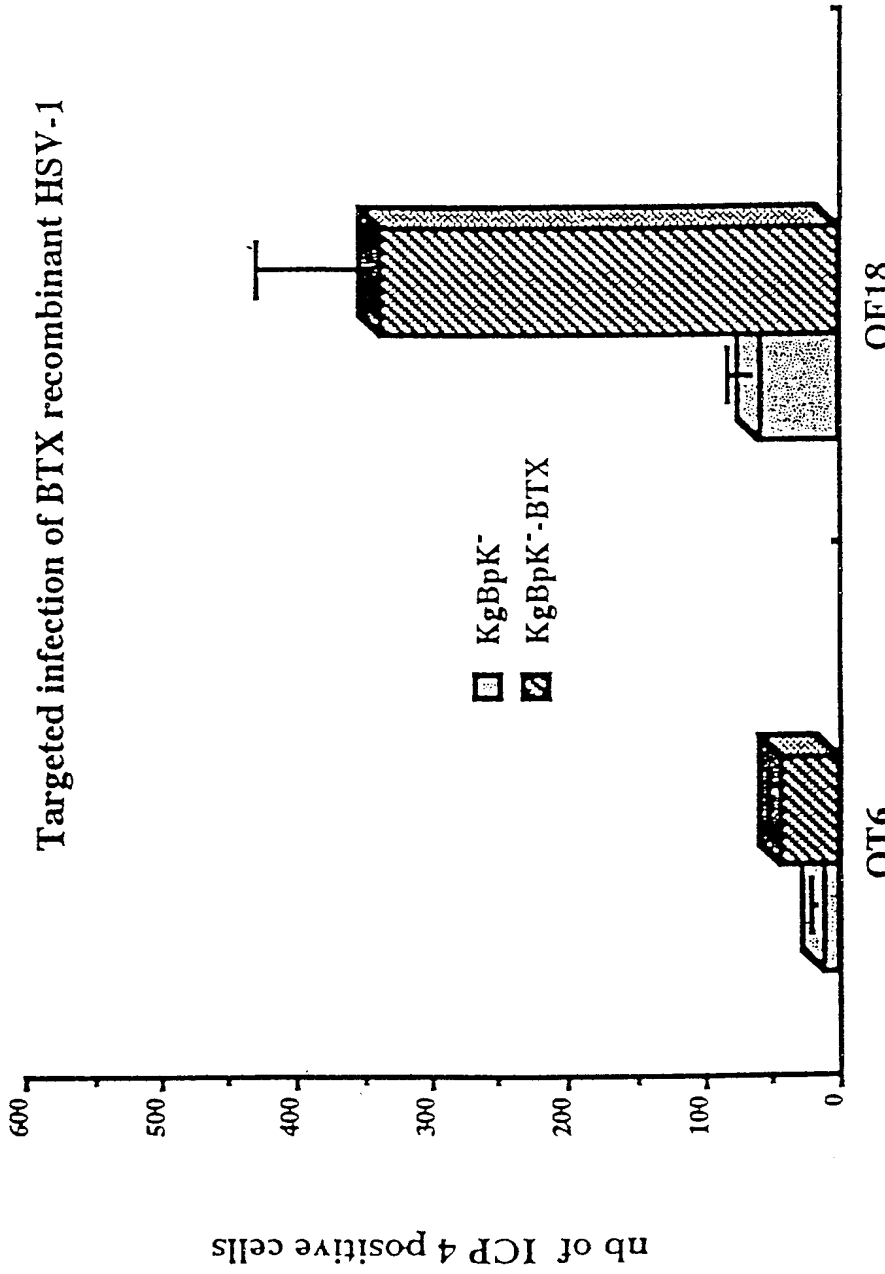
Targeted infection of BTX recombinant HSV-1
nb of ICP 4 positive cells
600
500
400
300
200
100
0
KgBpK-
KgBpK-BTX
QT6
QF18
Cell type

Fig. 7

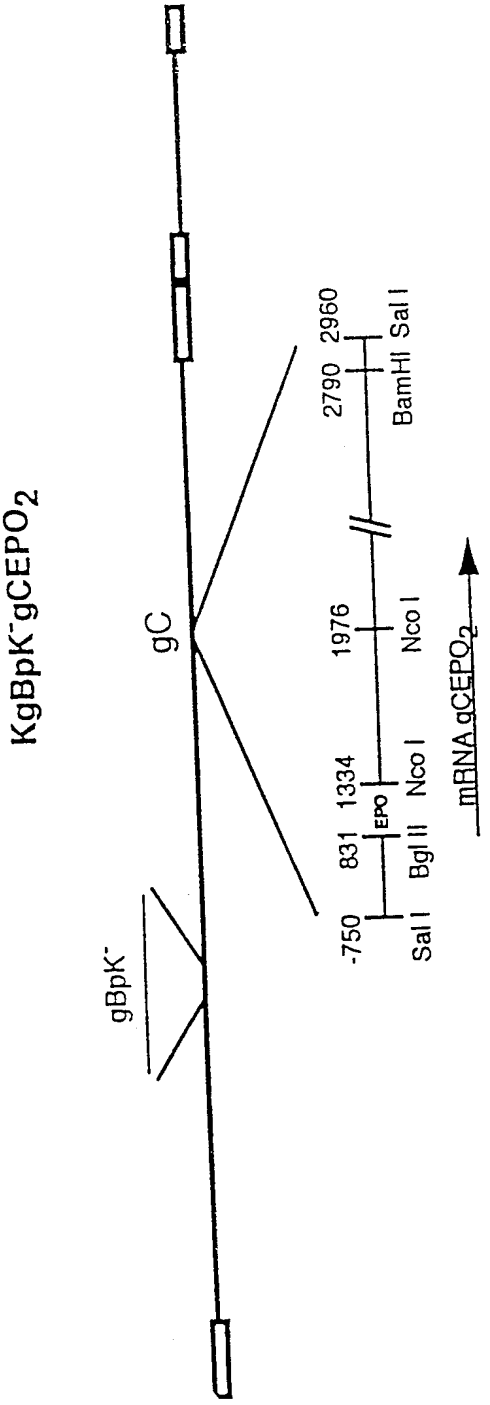
KgBpK⁻gCEPO2
gC
gBpK⁻
-750
831
1334
1976
2790
2960
Sal I
Bgl II
EPO
Nco I
Nco I
BamHI
Sal I
mRNA gCEPO2

Fig. 8

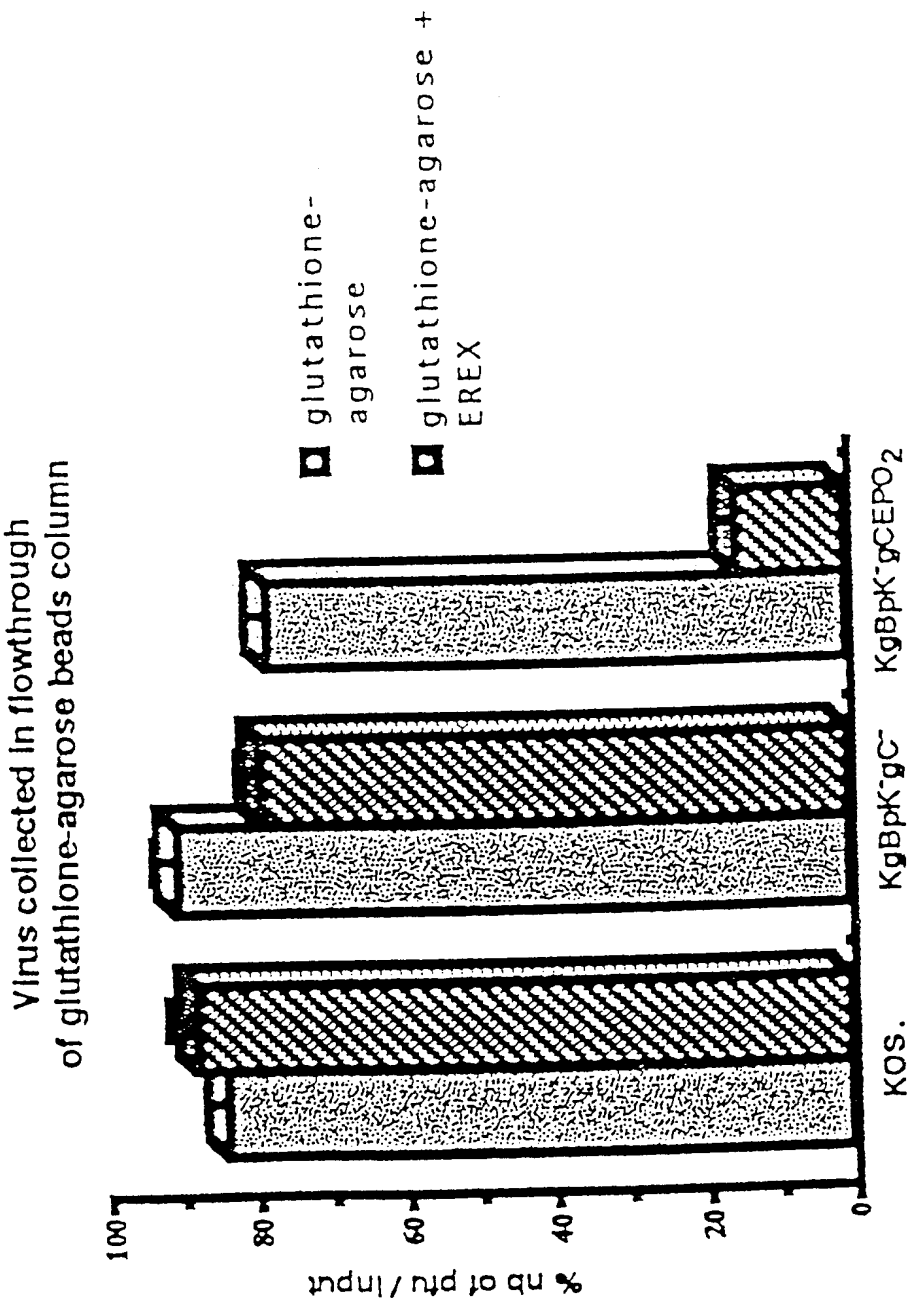
Virus collected in flowthrough
of glutathione-agarose beads column
glutathione-agarose
glutathione-agarose + EREX
% nb of pfu / input
100
80
60
40
20
0
KOS.
KgBpK⁻gC⁻
KgBpK⁻gCEPO2

Fig. 9

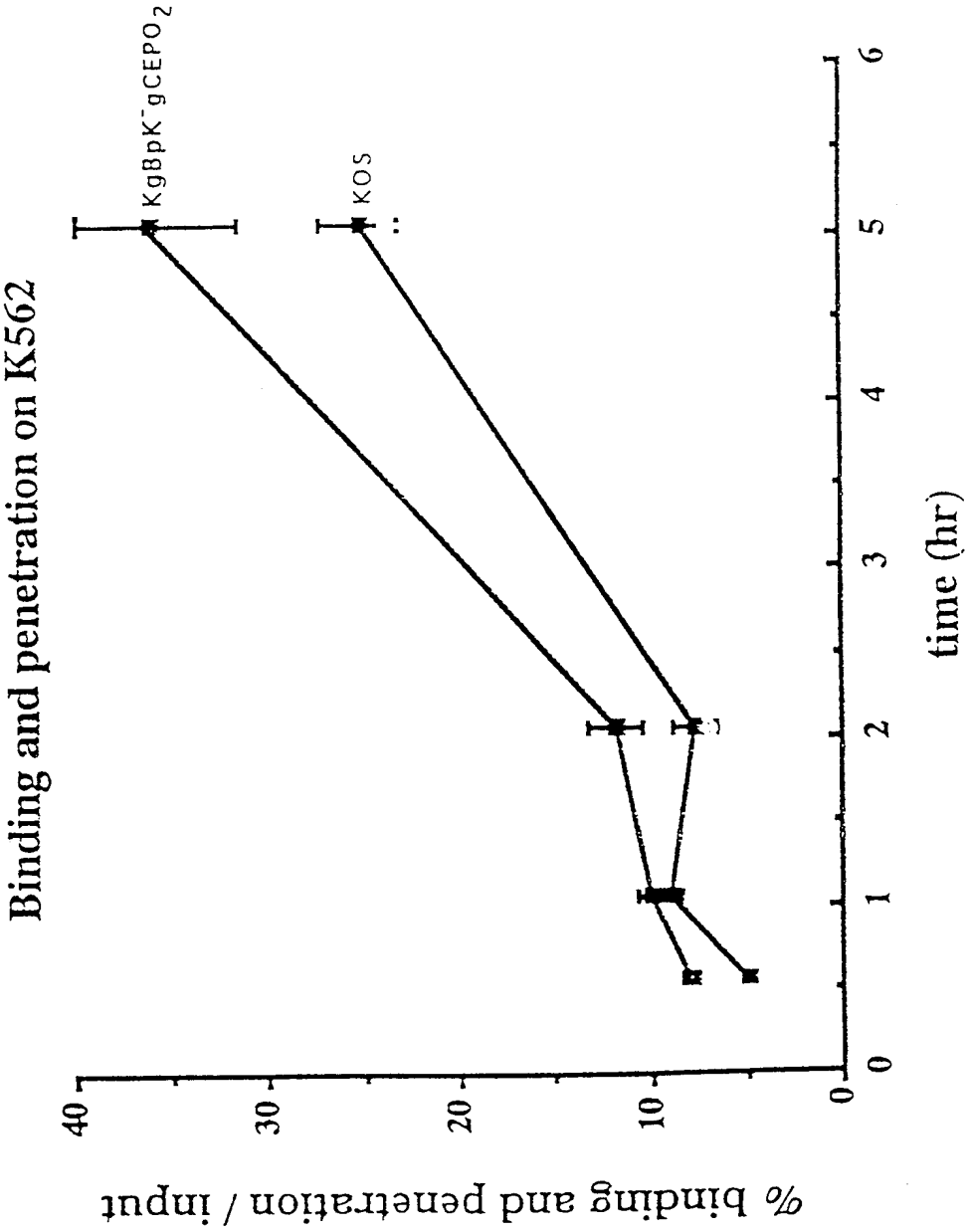
Binding and penetration on K562
KgBpK-gCEPO2
KOS
% binding and penetration / input
time (hr)
0
10
20
30
40
0
1
2
3
4
5
6

Fig. 10

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/16051

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 05266 A (INTROGENE BV ;VALERIO DOMENICO (NL); BEUSECHEM VICTOR WILLEM VAN () 13 February 1997 see page 3, line 17 - page 4, line 16	1-5, 7-12, 14-21
Y	see page 21, line 13 - line 17	6,13
Y	ROIZMAN B. AND SEARS A. E.: "Herpes simplex viruses and their replication. " 1990 , RAVEN PRESS , NEW YORK, FIELDS VIROLOGY, PAGES 1800-1801 XP002081536 see the whole document	6,13
A	WO 94 10323 A (IMP CANCER RES TECH ;SPOONER ROBERT ANTHONY (GB); EPENETOS AGAMEMN) 11 May 1994 see the whole document	1-21

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1998	05/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

1

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INTERNATIONAL SEARCH REPORT

In ,tional Application No
PCT/US 98/16051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KASAHARA N. ET AL.: "TISSUE-SPECIFIC TARGETING OF RETROVIRAL VECTORS THROUGH LIGAND-RECEPTOR INTERACTIONS" SCIENCE, vol. 266, no. 5189, 25 November 1994, pages 1373-1376, XP000541772 see the whole document	1-21

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/16051

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9705266 A	13-02-1997	AU 6535096 A	26-02-1997
		EP 0840797 A	13-05-1998
WO 9410323 A	11-05-1994	EP 0672158 A	20-09-1995
		GB 2286593 A,B	23-08-1995
		JP 8506239 T	09-07-1996